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Regulation of electron flux in the branched respiratory chain in mitochondria of *Acanthamoeba castellanii*

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Mitochondria of amoeba *A. castellanii* were found to share many features with mitochondria of higher plants [1]. These are: (i) coexistence of two constitutive pathways of electron transport, the phosphorylating one passing through the cytochrome chain, and the alternative one, nonphosphorylating and insensitive to KCN; (ii) the presence of a nonelectrogenic bypass for the electrons from the matrix pool of NADH to ubiquinone; (iii) the ability to oxidize exogenous NADH. Like in plant mitochondria [2], the KCN-insensitive alternative pathway of the amoeba branches from the main cytochrome chain at the step of ubiquinone [1]. In amoeba mitochondria, unlike in plant mitochondria, the activity of the alternative pathway is strongly stimulated by purine nucleoside 5'-monophosphates, especially by GMP. Another common feature is the oxidation of malate carried out by two matrix enzymes: malate dehydrogenase and NAD-dependent malic enzyme, which produce oxalacetate and pyruvate, respectively [3]. The mechanism by which the electron flux is partitioned into this complicated electron transport system requires precise regulation. Such a regulation is essential because the activity of the cytochrome and the alternative pathways is coupled to the production of different amounts of ATP.

Mitochondria were isolated from trophozoites of axenically grown A. *castellani* at the exponential phase of growth (at a density of about 2–4 × 10^6 cells/ml). O_2 uptake was measured polarographically in 2.7 ml of the medium (25°C, pH 7.4, 120 mM KCl, 20 mM Tris/HCl, 3 mM KH_2PO_4, 8 mM $MgCl_2$ and 0.2% (w/v) bovine serum albumin, with 1–2 mg of mitochondrial protein. Values of O_2 uptake presented in natom × min^{-1} per mg protein. NADH oxidation was monitored simultaneously with O_2 uptake, in an Aminco-Chance double-beam spectrophotometer (370/400 nm) in 1.7 ml of the medium. Membrane potential ($\Delta\Phi$) was measured together with the oxygen uptake in the same incubation mixture with a TPP^+-specific electrode [4].

The regulation of electron flux in the branched respiratory chain of amoeba mitochondria was studied using respiratory substrates which differ in the topology of the electron input into ubiquinone: 10 mM isocitrate or malate (NAD-linked substrates), 10 mM succinate, 1 mM NADH. In the absence of KCN, only succinate was oxidized simultaneously *via* the cytochrome and alternative pathways in state 4. This was proved by the inhibition of O_2 uptake by SHAM (an inhibitor of the alternative pathway) (Fig. 1A). Our results indicate that saturation of the cytochrome pathway with electrons is not necessary to engage the alternative pathway in total respiration, as postulated by Bahr & Bonner [5]. Although the activity of the alternative pathway was found with succinate oxidized separately and with

[1] Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TPP^+, tetraphenyl phosphonium; SHAM, salicylhydroxamic acid; UQ, ubiquinone; $\Delta\Phi$, transmembrane electrical potential difference (membrane potential).

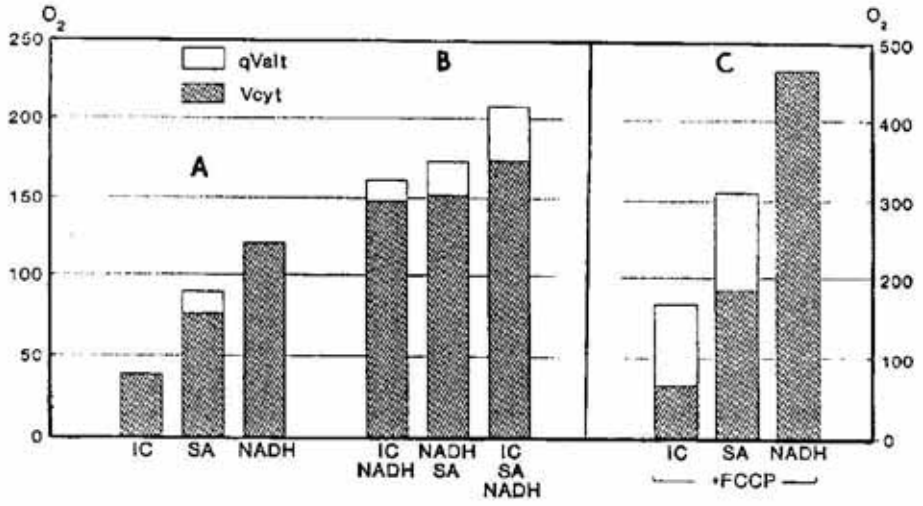

Fig. 1. Participation of the alternative pathway in total respiration in state 4 and in the uncoupled state.
A, Oxidation of single substrates; B, effect of combined substrates; C, effect of FCCP on the substrates oxidized separately. Note the difference in scale between panels A+B and C. Vcyt, the cytochrome-mediated respiration determined in the presence of SHAM; qValt, part of the alternative pathway capacity actually engaged in uninhibited respiration, determined as the difference between uninhibited respiration and the cytochrome pathway mediated respiration. Abbreviations: IC, isocitrate; SA, succinate.

combinations of two substrates (independently of the presence of succinate in a pair of oxidized substrates), saturation of the cytochrome pathway was observed only, when mitochondria oxidized three substrates simultaneously (i.e. isocitrate, succinate and NADH) (Fig. 1B). This is consistent with the suggestion of Day *et al.* [6] that some critical redox state of UQ is sufficient to "switch on" the alternative pathway. It is reasonable to expect that this redox level of UQ would be higher for NADH than for succinate.

In the case of isocitrate (a substrate oxidized slower than succinate engagement of the alternative pathway was found only in the presence of 1 µl FCCP which increased electron flux (thus, the degree of UQ reduction) (Fig. 1C). Under these conditions external NADH was still oxidized exclusively *via* the cytochrome pathway. Our results suggest that, in amoeba mitochondria, the activity of the alternative pathway in uninhibited total respiration depends not only on the respiratory rate (thus,

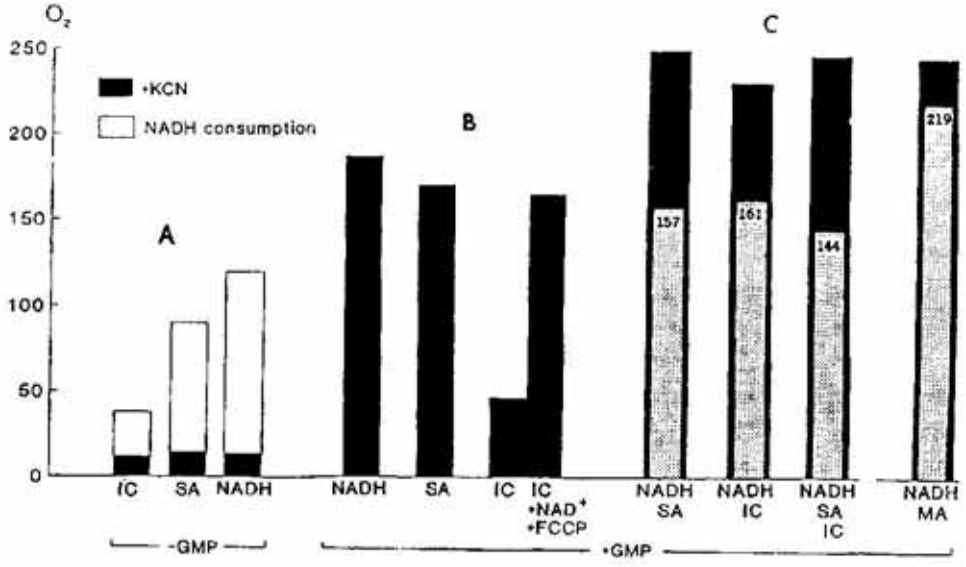

Fig. 2. Capacity of the unstimulated (A) and GMP-stimulated (B, C) alternative pathway in the presence of KCN.
Consumption of NADH (C), measured spectrophotometrically, is presented in nmol × min^{-1} per mg protein. Abbreviations: MA, malate; other abbreviations as in Fig. 1.

redox state of UQ) but also on the nature of the substrate. Like in plant mitochondria, electrons from external NADH, compared to those from citric acid cycle derived substrates (isocitrate, malate and succinate), seem to have higher (or exclusive) affinity to the cytochrome pathway [2, 6, 7]. In plant mitochondria, this situation is observed both under uninhibited and KCN-inhibited conditions. In contrast, in amoeba mitochondria, when the cytochrome pathway is excluded by KCN, electrons from external NADH have a similar access to the alternative oxidase as the electrons from isocitrate and succinate, irrespective of the presence or absence of a stimulator of the alternative pathway (GMP). This situation corresponds to lack of stimulation of NADH oxidation by isocitrate or/and succinate (Fig. 2C, spectrophotometric measurement), whereas in plant mitochondria this stimulation is of importance [6, 7]. In amoeba mitochondria, the stimulation of NADH oxidation by malate (Fig. 2C) seems to be only a result of additional consumption of NADH during reduction of oxalacetate by malate dehydrogenase in the intermembrane space. During oxidation of malate *via* the GMP-stimulated alternative pathway oxalacetate (beside pyruvate) is produced [3]. The results presented in Fig. 2A indicate that the rate of electron flux towards cytochrome oxidase depends on the activity of substrate dehydrogenases. On the other hand, the latter does not affect the alternative oxidase-mediated respiration which would be regulated by the capacity of the alternative pathway (+GMP), similar for all substrates. One can estimate participation of the electron flow from particular substrates in the oxidation of their combination and the competition for the access to the alternative pathway while investigating the level of $\Delta\Phi$ generated by site I in the presence of KCN and GMP (Fig. 3). This potential, generated by isocitrate is reduced by the addition of substrates introducing electrons directly at the level of UQ (succinate and NADH). Moreover a parallel spectrophotometric measurement indicates that the contribution of electrons from NADH in oxidation of the three substrates is about 60%, proving that NADH competes with them for electron input into the alternative pathway (Fig. 2). Investigation of the competition between substrates for feeding electrons to UQ is important for understanding of the physiological role of the alternative pathway because, presumably, mitochondria oxidize *in vivo* several substrates simultaneously. The difference between electrons from citric acid cycle derived substrates and NADH in access to the alternative pathway (under uninhibited conditions) suggests the existence in the amoeba, like in plant mitochondria [6, 7] of a separate UQ-pool for externally introduced electrons from NADH. On the other hand, in the presence of KCN the access to the alternative pathway is similar for electrons from all substrates. Further experiments are required to elucidate the existence of a single or heterogenous UQ-pool in amoeba mitochondria.

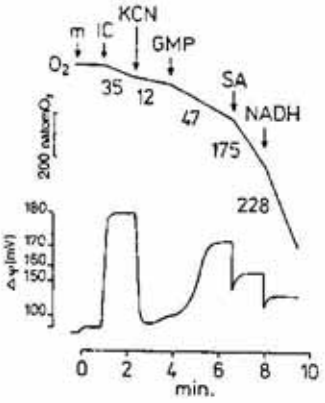

Fig. 3. Competition of electrons from different substrates for the alternative pathway reflected in O_2 uptake and the level of $\Delta\Phi$.
Abbreviations: m, mitochondria; other abbreviations as in Fig. 1.

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